Attachment #6

P07 43598 (CEDAR) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Readhead *et al.*	Art Unit:	1633
Serial No.:	09/191,920	Examiner:	D. Clark
Filed:	November 13, 1998		

For: **TRANSFECTION, STORAGE AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES & GENETIC THERAPIES**

considered
/W 12/27/01

DECLARATION OF DR. CAROL W. READHEAD

UNDER 37 C.F.R. § 1.132

1. I, Carol W. Readhead, residing at 2185 San Pasqual Street, Pasadena, CA 91107, declare that I have personal knowledge of the facts averred herein.

2. I am a co-inventor of the invention described and claimed in U.S. Patent Application Ser. No. 09/191,920 for TRANSFECTION, STORAGE AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES & GENETIC THERAPIES, claiming priority from U.S. Provisional Application No. 60/065,825, filed on November 14, 1997. Professor Robert Winston, who resides at 11 Denman Drive, London NW 11 6RE, United Kingdom, is a co-inventor. Together Professor Winston and I are the sole inventors of the claimed invention.

3. Professor Winston and I successfully introduced exogenous genetic material into male germ cells of mice, in accordance with the claimed in vivo method of incorporating a polynucleotide into a male vertebrate's germ cells. Subsequent to natural matings of the treated animals, we further obtained production of transgenic progeny that expressed the marker in their cells. These events are described in detail in the following paragraphs 4-8b.

4. Microsurgery. After depopulation of the testis, viral particles were delivered to the seminiferous tubules as follows: Mice were anaesthetised with isofluorane (0.5-2% in oxygen). Each testis was exposed through a midline abdominal incision. Using a microsurgical approach (Winston, R.M.L., *Microsurgical reanastomosis of the rabbit oviduct and its functional and pathological sequelae*, Brit. J. Obstet Gynaecol. 82:513 - 522 [1975]; Zeiss microscope at magnification 4 to 50x) the tissue bundle containing the vasa efferentia was visualised. Dissection from the surrounding fat was aided by a stream of phosphate buffered saline forced through a fine needle. A quartz glass micropipette was back-filled with 10 μL viral particles (10^9pfu/ml) mixed with 1 μL polybrene (80mg/mL). This was attached to a

micropipette (Eppendorf) and the particles introduced into the vas efferens under 2.2 bar pressure in pulses of 1.5 seconds, controlled by foot pedal. Earlier trials using 1% Bromophenol dye showed that most seminiferous tubules could be filled, but during treatments, no dye was used and small air bubbles were introduced into the liquid containing viral particles to confirm dispersion into the seminiferous tubules. To preserve fertility, only single vasa efferentia were injected, reducing injury to the remaining ducts.

5 Preparation of the Viral Vector. The plasmid, pHR'-CMVLacZ (Naldini, L., *et al., In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector*, Science 272: 263-267 [1996]), was modified by replacing the *Bam*HI – *Xho*I fragment containing the *LacZ* gene with a fragment containing the EGFP gene ('humanised' GFP, Clontech). For the production of viral particles 40 μg plasmid DNA was used to transfect a 10-cm plate of 293T cells. The 40 μg of plasmid DNA was made up of 10 μg pCMV R9, 20μg of modified pHR' and 10 µg envelope plasmid. Vesicular-stomatitis-virus-glycoprotein (VSV-G) pseudotyped vectors were produced by contransfection of the vector plasmid with the Moloney murine leukemia virus (MLV) gag-pol packaging plasmid pCMV-GAGPOL and the VSV-G plasmid. The supernatant was harvested 48-60 hours after transfection, subjected to high speed centrifugation, filtered through 0.45 µm filters and assayed. The transducing viral particles had the MLV restricted envelope protein, *env*, substituted with a broad-spectrum *env* protein from the vesicular stomatitis virus.

6 In Vivo Transduction of Male Germ Cells. Six mice were now treated with viral particles containing the transducing vector pHR' (10^9 particles/mL). A single vas efferens was injected with a volume of 10µL retroviral concentrate together with 1µL (80mg/mL) polybrene. After 24 days the mice were sacrificed and the testes removed and fixed for cryosectioning and histological examination. Testes were fixed for 48 hours in 4% Paraformaldehyde pH 7.4, and placed in 20% sucrose in phosphate saline buffer pH 7.4 at 4°C for 24 hours. They were embedded in OCT and stored at –70°C. They were cryosectioned at 8µm and viewed in a Zeiss 410 confocal microscope (Fig. 1). Nearly all tubules sectioned contained cells expressing GFP Expression was highest in Sertoli and spermatogonia cells (Fig. 1a -b).

7. Natural Matings with Females after Transduction of Male Germ Cells. Eleven C57/B1/6J young males were then selected to test whether transduced male germ cells could transmit the retrovirally integrated transgene to the next generation. Six of these mice were treated with a bolus of busulfan (IP; 4µg/gm body wt.) 14 days before in vivo transduction microsurgery in accordance with the in vivo method of incorporating exogenous genetic material into the genome of a vertebrate, as described above, and three received the same dose only one week before in vivo transduction. Two other mice were not pre-treated with busulfan before the in vivo transduction operation. Lentiviral particles were introduced into the seminiferous tubules. After 14 weeks, B6D2F1 females were introduced into cages with the males. Transduced males fathered at least two successive litters. Litters were conceived 14, 15, 19 and 20 weeks after transduction. All the males, except one dying immediately after surgery, fathered transgenic offspring. (Table 1, next page).

Table 1. Production of transgenic offspring per litter fathered by treated males at various times after mating.

Mouse #	Pre-treatment	14 weeks	15 weeks	19 weeks	20 weeks
1	Busulfan 1 week	-	2/9 (22%)	8/10 (90%)	0/9 (0%)
2	Busulfan 1 week	-	1/7 (14%)	1/7 (14%)	2/7 (28%)
3	Busulfan 1 week	4/7 (57%)	1/8 (12%)	4/6 (66%)	0/7 (0%)
4	Busulfan 1 week	7/8 (87%)	3/7 (43%)	1/6 (17%)	1/8 (12%)
5	Busulfan 2 weeks	5/6 (83%)	8/9 (89%)	-	0/8 (0%)
6	Busulfan 2 weeks	-	2/8 (25%)	8/8 (100%)	1/9 (11%)
7*	Busulfan 2 weeks	-	-	-	-
8	Busulfan 2 weeks	-	6/6 (100%)	-	1/8 (12%)
9	Busulfan 2 weeks	-	8/8 (100%)	-	3/10 (30%)
10**	none	2/5 (40%)	5/6 (83%)	-	-
11	none	3/7 (43%)	7/8 (88%)	-	0/6 (0%)

*Mouse No. 7 died immediately after surgery.

**Mouse No. 10 died 17 weeks after surgery.

8 PCR and Southern blot analysis of DNA from embryonic offspring. Embryos at approximately embryonic day 12.5, were screened for presence of the transgene by polymerase chain reaction (PCR) and Southern blot analysis. For the PCR, GFP specific primers were used and a radiolabeled GFP cDNA probe was used for the Southern blot analysis (Fig. 2). DNA was purified from embryos using the Gentra purification system. The presence of the transgene was ascertained using PCR amplification with the following GFP specific primers:

(A) forward primer: 5'-GGT GAG CAA GGG CGA GGA GCT-3'

(B) reverse primer: 5'-TCG GGC ATG GCG GAC TTG AAG A-3'

The PCR cycling conditions were: denaturing 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 3 minutes. PCR ran for 35 cycles and yielded a specific GFP product 470 base pairs in length. Each cycle step can be reduced to one second - "one second PCR" to yield a distinct 470-bp PCR amplification product. Southern blot analysis

was also done on the same embryo DNA extracts. The DNA was cut with *Bam*HI-*Xho*I, run on a 0.8% agarose gel and blotted overnight in 20x SSC onto Hydrobond XL paper. The blots were hybridised overnight at 65°C with a ^{32}P -radiolabeled *Bam*HI-*Xho*I GFP fragment isolated from the pHR'plasmid. The blots were washed at 65°C (30 minutes) each in 2x SSC with 0.1% SDS, 1x SSC with 0.1% SDS, 0.1 X SSC with 0.1% SDS and exposed to X-ray film.

8a. PCR and Southern analysis showed that a high percentage of transgenic offspring were obtained in litters conceived within 15 weeks. The results are summarized in Table 1. By 20 weeks the percentage of transgenic progeny had dropped in all of the treatment groups, implying that the self-renewing spermatogonia were not transduced, but rather a population of differentiating spermatogonia. Once the daughter cells from this population had matured and left the testis they were not renewed (Huckins, C. & Oakberg, W.F , *Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II. The irradiated testes*, Anat. Rec. 192:529-42 [1978]) In Table 1, the ratios are the number of transgenic offspring out of the total number of embryos in the litter.

8b. Although pre-treatment with busulfan enhanced the transduction of spermatogonia, mice untreated with busulfan also generated transgenic offspring. Male germ cells take 60 days to differentiate from spermatogonia (Russell, L.D., *et al.* In: *Histological and Histopathological evaluation of the testis*, Cache River Press [1990]), undergo meiosis and form spermatozoa. Since conception was more than 60 days after transduction, it is presumed that the transgenic offspring were conceived from differentiated daughter cells of transduced spermatogonia. EGFP expression was driven by the CMV promoter and was evident in the testicular cells of the founder males 24 days after infection. The animals that were infected did not appear to have toxic side effects with the possible exception of one dying 17 weeks after surgery.

Carol W. Readhead 11/14/00

Carol W. Readhead Date

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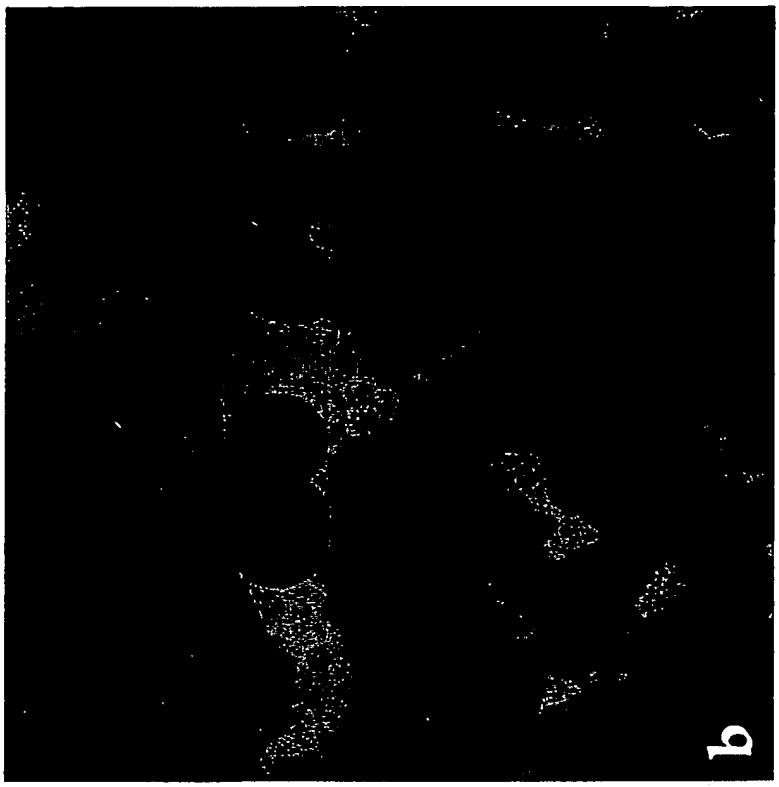
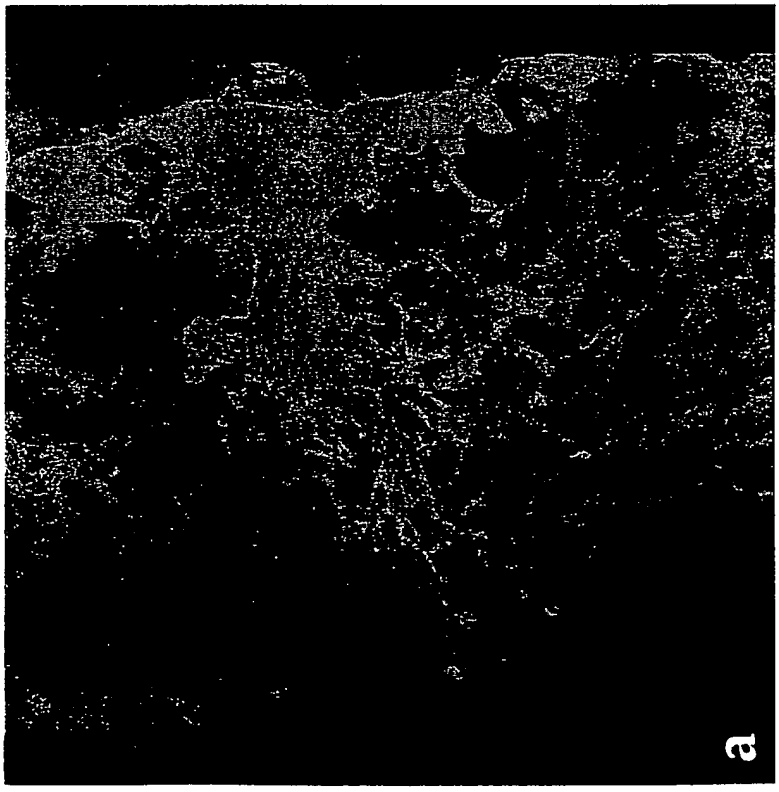
EXHIBIT A

Figure 1 shows testicular cells transduced by a pseudotyped lentiviral vector expressing Green Fluorescent Protein (GFP) in Zeiss 410 confocal images (wavelength 488 nm; 19 stacked images) of a cryosection of mouse testis. Figure 1(a) shows a transduced Sertoli cell expressing GFP. Figure 1(b) shows transduced spermatogonia; GFP expression is visible in the cytoplasm surrounding large dark nuclei.

Figure 1





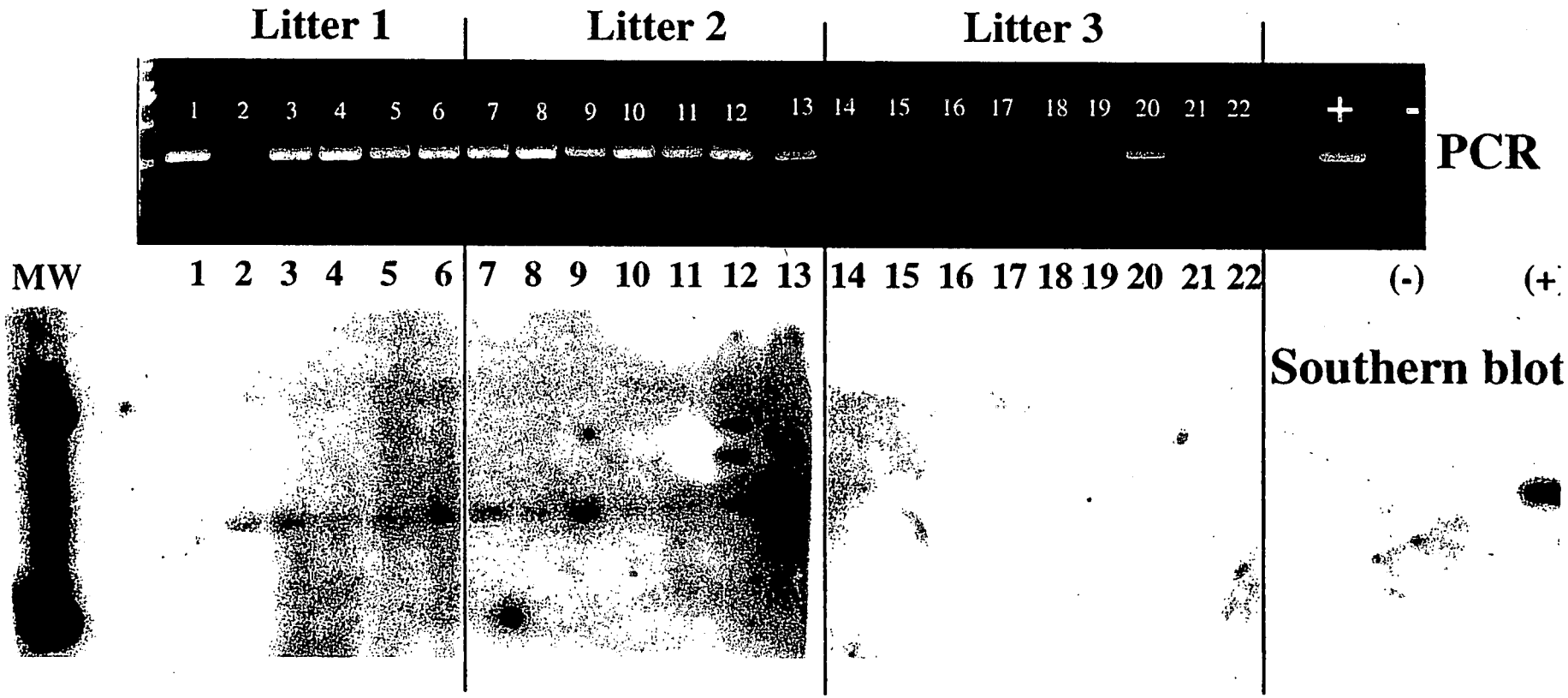

Litter 1
Litter 2
Litter 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 + -
PCR
MW
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 (-) (+)
Southern blot

Figure 2

Figure 2 shows a DNA analysis from three consecutive litters of progeny from one male treated in accordance with the in vivo method of incorporating exogenous genetic material into the genome of a vertebrate. The top panel shows GFP-specific PCR amplification products separated on an agarose gel from embryonic DNA of 22 individual progeny. In this run, there was an absence of amplification from fetus No. 2, but other PCR assays confirmed the presence of the transgenic reporter gene. The bottom panel shows a Southern blot analysis of the same DNA. The Southern blot was probed with a radiolabed GFP DNA fragment.

EXHIBIT B

symptoms, biochemical or physiological defects, behavior, or other phenotypes of interest. The resource provides technical support for users of JAX mice to answer questions regarding genetics, husbandry, and characteristics of mutant mice. All mice can be ordered by calling The Jackson Laboratory's Customer Service Department at 1-800-422-MICE. A fee for mice is charged to partially recover strain maintenance costs and shipping expenses. For more information about the resource, contact any of the four investigators listed above. Updates on strain availability and other information are accessible by Internet at: http://www.jax.org/resources/documen ts/mmr/.

The online form for submission of strains is available at http://www.jax.org/reso urces/documents/grc/grcspontout.html.

Mice

This resource maintains strains of mice with specific mutant genes in various categories, including growth and development, reproduction, neurological, neuromuscular, vision and hearing, skeletal, immunological, skin and hair, pigmentation, kidney, and enzyme deficiencies. It also maintains stocks of mice with chromosomal aberrations including inversions, translocations, monosomy, and trisomy. In addition, several wild-derived inbred strains are maintained for linkage crosses. Details of the mouse strains available from The Jackson Laboratory are accessible by Internet at http://www.jax.org/resources/docu ments/pricelist/ or http://jaxmice.jax.org.

Index Terms

Genetic diseases, genetics, mouse, mouse models, mutations.

National Repository for Transgenic Mice and Rats

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Research Emphasis/Objectives

The objective of this repository is to make transgenic mouse and rat models of high health quality available to investigators. Current research efforts focus on the cryopreservation of sperm and recovery of offspring from frozen and thawed spermatozoa.

Resources Provided

The National Repository for Transgenic Mice and Rats is an integral part of the Transgenic and Targeted Mutant Repository or Induced Mutant Resource (IMR) at The Jackson Laboratory. The function of the IMR is to select, import, cryopreserve, maintain, and distribute

these important strains of mice and rats to the research community. To improve their value for research the IMR also undertakes genetic development of stocks, such as transferring mutant genes or transgenes to defined genetic backgrounds and combining transgenes and/or targeted mutations to create new models for research.

Included in the repository are cancer, immunological, neurological, behavioral, cardiovascular/heart, developmental, metabolic, and other models. A list of all strains may be obtained from the IMR Web site: http://www.jax.org/resources/ documents/imr/notes.html.

In addition to serving as a repository for mouse and rat strains, the National Repository for Transgenic Mice and Rats offers to store and distribute cryopreserved embryonic stem (ES) cell lines carrying targeted mutations.

Index Terms

Cryopreservation, disease models, transgenic mice, transgenic rats.

Transgenic Mice With Altered Calcium Handling

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URL: http://blues.fd1.uc.edu/~kraniaeg/P 40_Grant1.html

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Research Emphasis/Objectives

The recent development of phospholamban knockout and phospholamban overexpression mice has revealed that phospholamban is a major regulator of basal contractility in the mammalian cardiac, smooth, and skeletal muscles. The regulatory effects of phospholamban are mediated through the Ca^{2+}-ATPase in sarcoplasmic reticulum (SERCA2), the key enzyme involved in muscle relaxation. Dephosphorylated phospholamban is an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase activity, and phospholamban relieves this inhibition. The overall research hypothesis is that alterations in the levels of the sarcoplasmic reticulum Ca^{2+}-ATPase or phospholamban, and the phosphorylated states of phospholamban are associated with alterations in calcium homeostasis and function of the muscles. Thus, the long-range research goal of this resource is to generate animal models with altered expression in each of these two key Ca^{2+}-cycling proteins; and altered expression of phospholamban phosphorylation mutants in cardiac, smooth, and slow-twitch skeletal muscle. These mouse models will be made available to the biomedical community at large to carry out further in-depth studies and to elucidate the mechanisms underlying intracellular calcium regulation and physiological responses in health and disease.

Resources Provided

This resource generates and maintains mouse models with genetic alterations in either phospholamban or the sarcoplasmic reticulum Ca^{2+}-ATPase in cardiac, smooth, or slow-twitch skeletal muscle. These models are initially characterized in the resource and then they are made available to interested investigators in the scientific community. A list of all current models may be obtained from the Sarcoplasmic Reticulum Mutant Mouse Resource (SR-MMR) Web site.

Index Terms

EXHIBIT C

1: Mamm Genome 2001 Aug;12(8):575-81

Trans-NIH neuroscience initiatives on mouse phenotyping and mutagenesis.

Moldin SO, Farmer ME, Chin HR, Battey Jr JF.

Genetics Research Branch, Division of Neuroscience and Basic Behavioral Science, National Institute of Mental Health, National Institutes of Health (NIH), 6001 Executive Blvd., Room 7189, MSC 9643, Bethesda, Maryland 20892-9643, USA.

In the post-genomic era, the laboratory mouse will excel as a premier mammalian system to study normal and disordered biological processes, in part because of low cost, but largely because of the rich opportunities that exist for exploiting genetic tools and technologies in the mouse to systematically determine mammalian gene function. Many robust models of human disease may therefore be developed, and these in turn will provide critical clues to understanding gene function. The full potential of the mouse for understanding many of the neural and behavioral phenotypes of relevance to neuroscientists has yet to be realized. With the full anatomy of the mouse genome at hand, researchers for the first time will be able to move beyond traditional gene-by-gene approaches and take a global view of gene expression patterns crucial for neurobiological processes. In response to an action plan for mouse genomics developed on the basis of recommendations from the scientific community, seven institutes of the National Institutes of Health (NIH) initiated in 1999 a mouse genetics research program that specifically focused on neurobiology and complex behavior. The specific goals of these neuroscience initiatives are to develop high-throughput phenotyping assays and to initiate genome-wide mutagenesis projects to identify hundreds of mutant strains with heritable abnormalities of high relevance to neuroscientists. Assays and mutants generated in these efforts will be made widely available to the scientific community, and such resources will provide neuroscientists unprecedented opportunities to elucidate the molecular mechanisms of neural function and complex behavior. Such research tools ultimately will permit the manipulation and analysis of the mouse genome, as a means of gaining insight into the genetic bases of the mammalian nervous system and its complex disorders.

PMID: 11471049 [PubMed - in process]

EXHIBIT D

1: Curr Opin Neurol 2001 Aug;14(4):441-7

Progress in the modeling of neurodegenerative diseases in transgenic mice.

Duff K, Rao MV.

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Transgenic mouse models exist for the major neurodegenerative diseases, including Alzheimer's disease, tauopathy and amyotrophic lateral sclerosis. Although many of the mice do not completely replicate the human disease they are intended to model, they have provided insight into the mechanisms that underlie disease etiology. In the case of the Alzheimer's disease and amyotrophic lateral sclerosis models, the mice have also provided a therapeutic testing ground for the testing of agents that have been shown to have considerable clinical promise.

Publication Types:
Review
Review, tutorial

PMID: 11470959 [PubMed - indexed for MEDLINE]

EXHIBIT E

1: Biochem Soc Symp 2001;(67):195-202

Transgenic mouse models of Alzheimer's disease: phenotype and mechanisms of pathogenesis.

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A range of transgenic mice have been created to model Alzheimer's disease. These include mice expressing human forms of the amyloid precursor protein, the presenilins and, more recently, tau. Several of the models develop features of the disease including amyloid pathology, cholinergic deficits, neurodegeneration and cognitive impairment. Progress in the characterization and use of these model animals is discussed.

PMID: 11447835 [PubMed - in process]